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LIFETIME REPRODUCTIVE PERFORMANCE OF MICE EXPOSED AS EMBRYOS TO X-IRRADIATION [1]

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In previous studies by Nash and Gowen (1962, 1965) it was shown with mice that following X-irradiation *in utero* postnatal growth and lifespan were dependent on both the amount of irradiation and the embryological age at the time of irradiation. In addition to the above characteristics, lifetime fecundity and fertility have also been studied in the same mice to evaluate further the long-term effects of embryonic and fetal irradiation. The present paper deals with those changes induced in the developing reproductive system as reflected in total lifetime reproductive performance.

In addition, examination of important genotypic-environmental interactions was investigated by studying the reproductive responses of several genetic backgrounds through the use of inbred strains of mice and the hybrids derived from crossing them.

Exposure of the mammalian embryo to ionizing radiations exposes rapidly dividing cells during periods of organ formation and differentiation. Many studies on the effects of acute irradiation on mammalian prenatal development have established that the type of developmental malformation is dependent on both the level of irradiation and the embryological stage at the time of treatment (reviews by Russell, 1954; O'Brien, 1956). The great majority of these studies has been concerned with those changes that have been evident within a short time of irradiation. Investigations of the long-term sequelae of embryonic or fetal irradiation have been somewhat limited.

Materials and Method

Three inbred strains of mice and the six possible types of hybrids derived from them were utilized in these investigations. The Committee on Mouse Nomenclature has designated the strains as BALB/Gw, K, and S. The three strains were chosen from those that were available at the Genetics Laboratory of Iowa State University at the time and represent a wide range of the spectrum of radiation response, the BALB and K strains representing relatively susceptible strains and the S strain representing a relatively resistant strain. The strains were differentiated originally by resistance to mouse typhoid (Gowen, 1948), but are known to differ in a number of other physiological characteristics including differences in response to various effects of irradiation (Grahn, 1954a, 1954b; Gowen and Stadler, 1956; Nash and Gowen, 1962, 1965; Stadler and Gowen, 1957a, 1957b).

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Matings within and among the three inbred strains of mice were set up. Females were examined daily for the presence of a vaginal plug, the plug being the sole criterion used to time the period of gestation and the approximate age of the embryos at the time of irradiation. The time of 4:00 AM was used as an approximate time of fertilization since it has been demonstrated by Snell (1940) that in the Bagg strain of mice, at least, fertilization occurred shortly after liberation of the egg which took place most often between midnight and 3:00 AM. Thus, the afternoon of the day on which a plug was observed was considered to be day $\frac{1}{2}$.

Pregnant females were exposed to a single whole-body dose of X-rays on day $6\frac{1}{2}$, $10\frac{1}{2}$, $14\frac{1}{2}$, or $17\frac{1}{2}$. Only first litters were used throughout these studies. The source of irradiation was a General Electric Maxitron which operated at 250 pkv, 30 ma with 0.25 mm Cu + 1 mm Al filtration at a distance of 50 cm from anode to mid-mouse. The dose rate was approximately 133 r/minute, the dosage rates having been measured in air by means of a rate meter. Pregnant mice were exposed within a circular, wooden container, $6\frac{1}{2}$ inches in diameter, and 1 inch in depth. The base of the container was $\frac{1}{4}$-by $\frac{1}{4}$-inch wire mesh, and the top was covered with two layers of cellophane. Four levels of irradiation were employed: 20, 80, 160, and 320 roentgens as well as nonirradiated controls.

Litters were recorded within 12 hours of birth and postnatal growth followed through 75 days of age (Nash and Gowen, 1962). All litters were weaned at 30 days, and the males and females separated at this time. At 75 days of age mice, irradiated or nonirradiated, were individually mated to nonirradiated mice of the Z strain. Previously unmated Z males or females were used in these matings. The treated animals were kept mated to Z mates for the remainder of their lives. Within each radiation-dose–embryological-age treatment, a total of two males and two females of each inheritance type was sought. It was not possible to have a completely orthogonal design due to mortality within some of the experimental groupings. For example, of the mice treated with 320 r at $6\frac{1}{2}$ days, or with 160 r and 320 r at $10\frac{1}{2}$, none survived until maturity. When a Z mate died before its treated partner, it was replaced within a few days by another virgin Z mouse of an age between two and six months.

All matings were observed daily and the following information taken: (a) total number of litters, (b) number of progeny within litters, (c) birth weights of individuals, (d) viability of mice within litters from birth to 21 days. Females were allowed to raise their litters, and the males were in the cages continually so that post-partum matings were possible. The mice were maintained in a well ventilated room, in which the environment was relatively constant. Food and water were available *ad libitum*. Additional data from these experiments describing effects on growth and life-span are provided in several papers including Nash and Gowen (1961, 1962, 1963a, 1963b, 1965).

Results

Of the various treatment combinations, only irradiation at day $10\frac{1}{2}$ with a dose of 80 r and above produced any gross congenital malformations. The types of malformations were similar to those reported in the mouse by other workers. None of the mice that were grossly malformed survived beyond the first day of

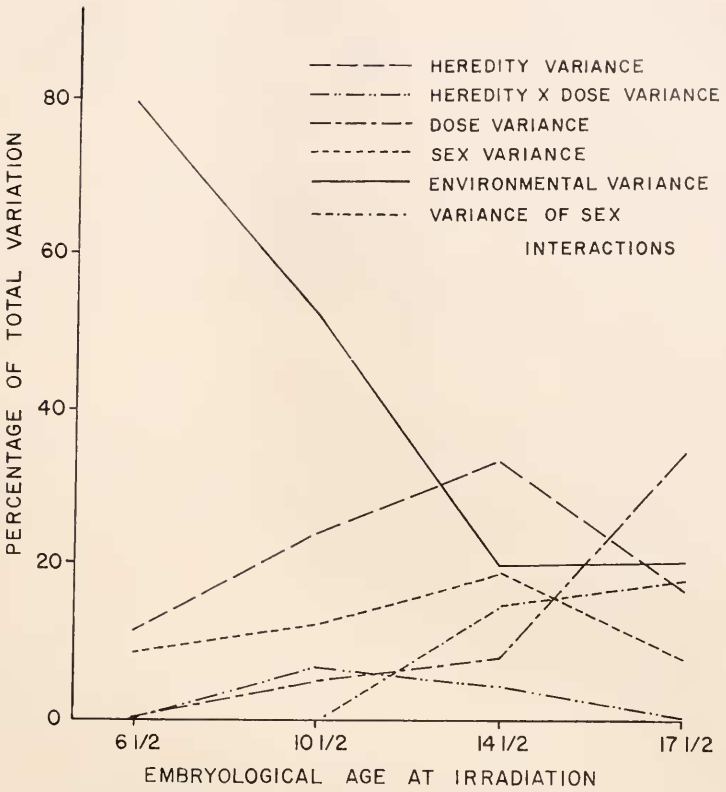

FIGURE 1. Contributions to variance of lifetime production of numbers of litters following in utero exposures to specific x-ray dosages received at different stages in embryological development.

life. Postnatal survival also was observed to be dependent on the level of irradiation and the embryological age at the time of irradiation. The mice that were subsequently tested for reproductive performance thus represent a sample of mice that had exhibited no congenital malformations and had survived to 75 days of age.

The number of sterile matings and the mean number of litters and progeny produced by males and females are shown in Table I. The overall incidence of sterile matings was low being pronounced only following irradiation at 17½ days with 160 r and 320 r, which produced 14% and 67% sterile matings, respectively. Following these two treatments 47% of the males were sterile and 33% of the females.

For both number of litters and number of progeny, similar analyses have been made. It is possible to arrange portions of the data to retain the statistical principle of orthogonality, but it does require a series of separate analyses. This has been accomplished by doing a separate analysis of variance for each of the embryological ages and for each of the levels of irradiation (Tables II and III).

Examination of the means for number of litters and number of progeny reveals that adult reproductive performance following embryonic irradiation is related both to the age of the embryo when irradiated and the level of irradiation.

Irradiation at 6½ days

Irradiation at 6½ days with doses up to 160 r apparently had little effect on subsequent fertility. Lack of effect on embryos that have been irradiated at this age but survive to be born have previously been noted for growth (Nash and Gowen, 1962) and lifespan (Nash and Gowen, 1965). Analysis of variance of these data revealed that there were significant differences due to genotype and sex, but not due to level of irradiation.

Irradiation at 10½ days

Embryos that had been exposed to 160 r or 320 r were stillborn or died within a few days of birth, so that the dose of 80 r was the highest dose tested in the reproductive studies. Although 80 r reduced the number of litters and progeny, the difference was not significant. Strain and sex effects were observed to be highly significant.

Irradiation at 14½ days

Only three embryos survived to maturity after 320 r and all were sterile. In addition to significant strain and sex effects, a significant treatment effect also

TABLE I

Incidence of sterility and mean number of litters and progeny produced in lifetime matings

Embryological age at irradiation	Irradiation dose	Females			Males		
		Number sterile	Mean number litters	Mean number of progeny	Number sterile	Mean number litters	Mean number of progeny
Control	0 r	2	9.1	79	0	12.3	94
6½ days	20 r	0	8.8	78	0	12.1	98
	80 r	0	7.7	68	0	12.0	93
	160 r	0	10.2	79	0	11.4	81
10½ days	20 r	0	8.0	77	0	12.5	94
	80 r	0	6.9	56	0	10.2	76
14½ days	20 r	0	9.3	74	0	12.8	95
	80 r	1	7.5	71	1	13.3	96
	160 r	3	6.1	45	0	10.8	80
17½ days	20 r	1	8.9	73	0	14.4	94
	80 r	0	7.6	62	0	13.2	97
	160 r	1	6.5	48	4	14.1	96
	320 r	11	2.9	24	13	2.3	12

TABLE II

Analysis of variance of number of litters produced in lifetime matings

Source of variation	Embryological age in days at exposure to irradiation							
	6½		10½		14½		17½	
	df	M.S.	df	M.S.	df	M.S.	df	M.S.
Dose (T)	3	6	2	33	3	67**	4	520**
Strain (G)	8	61*	8	71**	8	143**	8	138**
T × G	24	22	16	25	24	16	32	26
Sex (F)	1	380**	1	436**	1	803**	1	580**
T × F	3	19	2	11	3	10	4	89**
G × F	8	26	8	18	8	16	8	57**
G × T × F	24	22	16	10	24	18	32	24
Unaccounted for (E)	72	23	54	23	72	15	90	18

* 5% level of significance.
** 1% level of significance.

TABLE III

Analysis of variance of number of progeny produced in lifetime matings

Source of variation	Embryological age in days at exposure to irradiation							
	6½		10½		14½		17½	
	df	M.S.	df	M.S.	df	M.S.	df	M.S.
Dose (T)	3	531	2	3,851	3	5,545**	4	34,393**
Strain (G)	8	4,772*	8	5,454**	8	10,132**	8	10,224**
T × G	24	1,397	16	1,820	24	1,014	32	1,191
Sex (F)	1	11,025*	1	10,760**	1	25,229**	1	20,416**
T × F	3	1,028	2	124	3	1,158	4	2,881*
G × F	8	1,846	8	1,238	8	1,048	8	2,165
G × T × F	24	1,300	16	354	24	1,076	32	1,692
Unaccounted for (E)	72	2,250	54	1,448	72	694	90	1,137

* 5% level of significance.
** 1% level of significance.

was observed. Although exposure to doses through 80 r did not appear to affect reproduction, the dose of 160 r reduced number of litters to 79% and number of progeny to 72% of control values.

Irradiation at 17½ days

Sufficient progeny survived all levels of irradiation at 17½ days to enable all experimental subclasses to be tested for reproductive performance. Among the females, there was a decrease in the number of litters and progeny following all levels of irradiation. Among the males, fertility appeared normal through doses including 160 r, but dropped markedly after 320 r. The analysis of variance

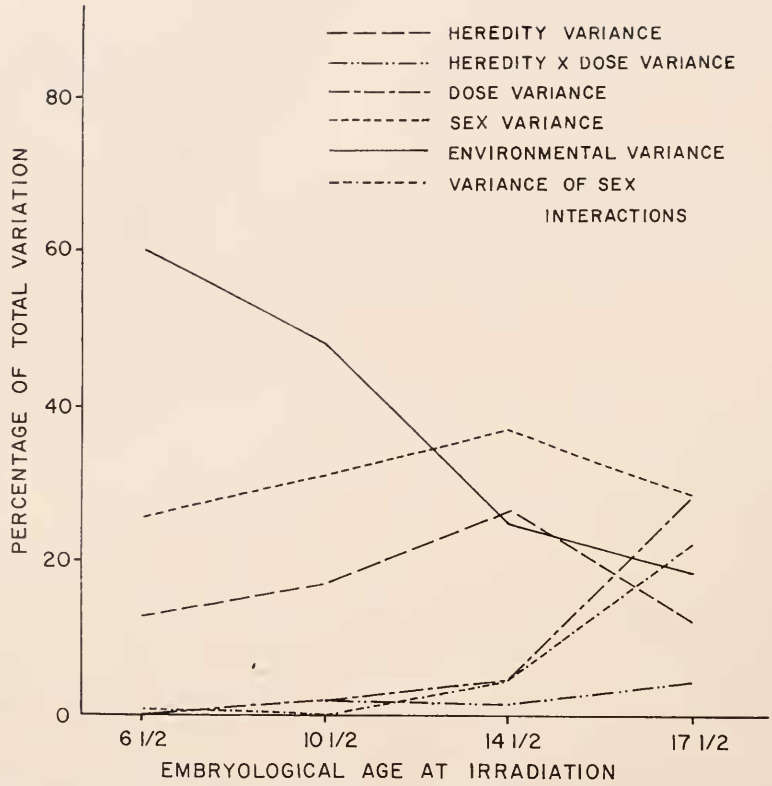

FIGURE 2. Contributions to variance of lifetime production of numbers of progeny following in utero exposures to specific x-ray dosages received at different stages in embryological development.

indicated that all three main effects, dose, strain, and sex were significant. In addition the Dose × Sex interaction was significant for both measures, and the Strain × Sex interaction was significant for number of litters. Concerning responses of specific genotypes, it was noted that after 320 r all of the inbred progeny were sterile, but only 50% of the hybrid progeny were sterile.

In addition to the analyses above, the data may be arranged to quantitate the effects of treatment at the different embryological ages when the levels of irradiation are fixed at 20 r, 80 r, and 160 r. Results of such analyses are given in Tables IV and V. Within the doses of 20 r and 80 r only the strain and sex effects are significant. Within 160 r, in addition to the significant strain and sex effects, there was a significant effect due to embryological stage and a significant Strain × Age interaction.

Estimation of components of variation

The amount of variation in lifetime reproductivities, as measured by number of litters and number of progeny can be partitioned into the amounts due to the

TABLE IV

Adult reproductive performance as influenced by the embryological age when the mice were irradiated; number of litters

Source of variation	Dose of irradiation in roentgens					
	20 r		80 r		160 r	
	df	M.S.	df	M.S.	df	M.S.
Embryological age (U)	3	11	3	22	2	85*
Strain (G)	8	94**	8	47*	8	145**
U × G	24	12	24	27	16	35*
Sex (F)	1	94**	1	779**	1	416**
U × F	3	14	3	11	2	52
G × F	8	23	8	40	8	30
G × U × F	24	30	24	26	16	23
Unaccounted for (E)	72	20	72	23	54	18

* 5% level of significance.
** 1% level of significance.

TABLE V

Adult reproductive performance as influenced by the embryological age when the mice were irradiated; number of progeny

Source of variation	Dose of irradiation in roentgens					
	20 r		80 r		160 r	
	df	M.S.	df	M.S.	df	M.S.
Embryological age (U)	3	106	3	1,625	2	4,878
Strain (G)	8	8,620**	8	4,298**	8	5,943**
U × G	24	675	24	1,538	16	2,691
Sex (F)	1	16,385**	1	24,258**	1	16,109**
U × F	3	248	3	358	2	3,803
G × F	8	1,410	8	1,100	8	834
G × U × F	24	1,149	24	1,518	16	1,847
Unaccounted for (E)	72	2,051	72	1,185	54	1,852

* 5% level of significance.
** 1% level of significance.

various effects and their interactions by utilizing estimated components of variance derived from an analysis of variance. It is assumed that the variations are due to components acting additively. The general mathematical model upon which the component analysis is based is:

$$Y_{ijkl} = u + g_i + t_j + (gt)_{ij} + f_k + (fg)_{ik} + (ft)_{jk} + (fgt)_{ijk} + e_{ijkl}$$

where u = the overall mean; $i = 1, 2, \ldots, 9$, the inheritance types; $j = 1, 2, 3,$ (or 4 or 5), levels of irradiation; and $k = 1, 2$, the sexes exposed to irradiation. The general breakdown for the component analysis is given in Table VI. The components can be interpreted as follows:

G is the variation due to genetic or hereditary differences, T is the variation due to differences in effects of the dosage levels, and F is the variation between sexes. The interaction terms are interpreted as arising from the differential responses of the geno-types or sexes from one level of irradiation to the next. The term E, is considered due to uncontrollable variation, and represents random variation of individual differences of mice of the same sex within a litter given the same treatment. A separate component analysis was obtained for each of the embryological ages. Results are shown in Figures 1 and 2 for number of litters and number of progeny, respectively, where graphs showing the contributions of the different components of variance are plotted on the embryological ages when the irradiations were administered. Except following irradiation at $17\frac{1}{2}$ days, the interactions involving sex: FG, FT and FGT, are small and have been combined within each of the embryological ages.

TABLE VI

Breakdown for the statistical analysis

Source of variation	d.f.	Components of variation
Among genotypes	8	$E + 2_jG$
Among dosages	(j-1)	$E + 18T$
Genotype × dosage	8(j-1)	$E + 2GT$
Between sexes	1	$E + 9_jF$
Sex × genotype	8	$E + {}_jFG$
Sex × dosage	(j-1)	$E + 9FT$
Sex × genotype × dosage	8(j-1)	$E + FGT$
Unaccounted for variation		E

* j = 3, 4, or 5, depending on which embryological age is analyzed.

The heredity influences on variance averaged slightly over 20% of the total variance for number of progeny and slightly under 20% for number of litters. The largest genotypic effects were observed following treatment on $14\frac{1}{2}$ or $10\frac{1}{2}$ days.

The relative importance of the dosage of irradiation components showed a slight increase from day $6\frac{1}{2}$ through day $14\frac{1}{2}$ increasing with increasing embryological age and reaching a maximum on day $17\frac{1}{2}$ when approximately 30% of the total variance was accounted for by treatment effect. It should be stressed, however, that the age of $17\frac{1}{2}$ days was the only stage in which mice survived a dose of 320 r for reproductive testing.

The interaction components between the levels of irradiation and genotypes on the different embryological ages were slight and never accounted for more than 10% of the total variation. The largest contribution to the variation of this interaction occurred on day $17\frac{1}{2}$. The highly specific effects between the genotype and the ability of the treated fetuses to carry out adult reproductive activities was noted early in the observation that none of the inbreds that had been exposed to 320 r had any litters at all.

The sex components showed variations at all embryological ages averaging 12% of the total variation in number of litters over all embryological ages and 27% of the variation in number of progeny. The basic sex differences in reproduc-

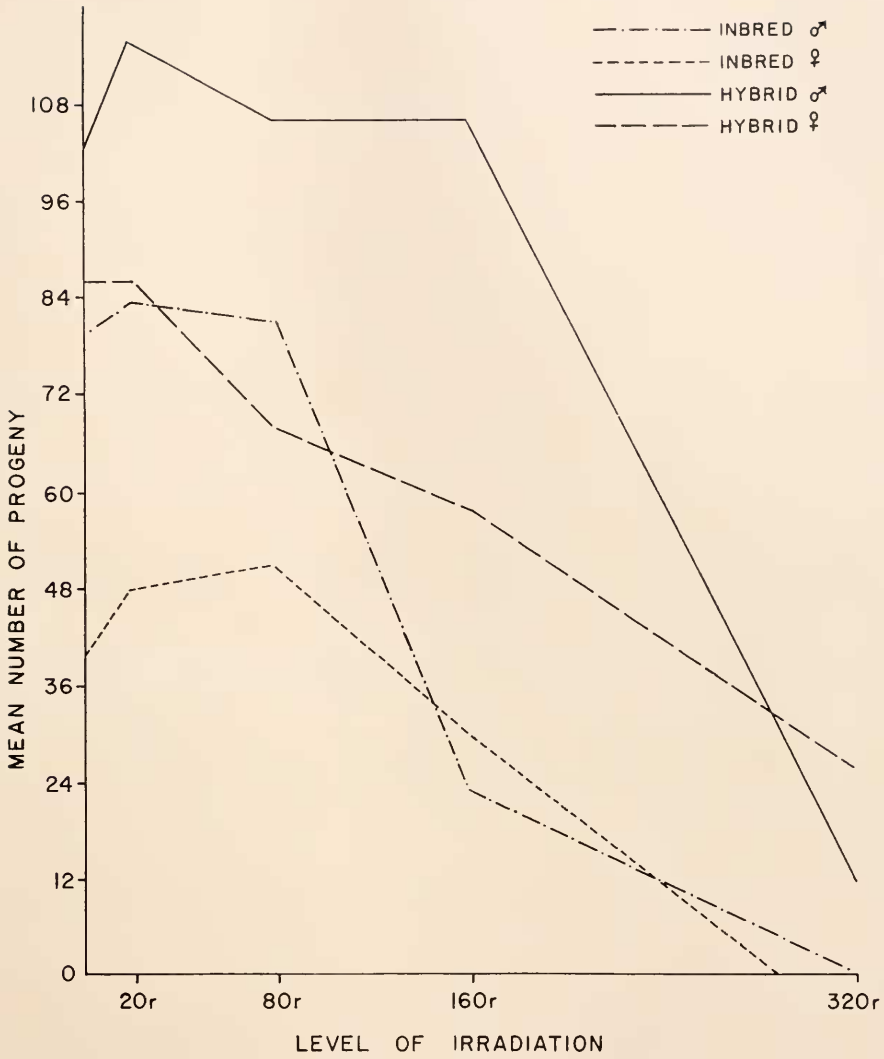

FIGURE 3. Mean number of progeny produced by inbred and hybrid mice following in utero exposures to specific x-ray dosages on day 17½ of gestation.

tive capacity are operational regardless of whether exposure to irradiation has occurred early or late in embryological development.

DISCUSSION

The results presented above demonstrate that embryological stage at the time of exposure to x-rays is most important in determining postnatal reproductive performance. Similar observations have been noted for other postnatal sequelae of embryonic irradiation. Although animals may appear normal at

birth following embryonic irradiation, effects on morphological and physiological characteristics may become evident in later life. Effects of prenatal irradiation upon postnatal growth have been reported for several species, including the mouse (Russell, Badgett and Saylors, 1960: Nash and Gowen, 1962; Rugh, Duhamel, Chandler and Varma, 1964), the rat (Ershoff and Bavetta, 1958), and cattle (Parish, Murphree and Hupp, 1962).

Understanding of results of prenatal irradiation upon adult reproductive performance can be aided by a consideration of the events that are taking place at each of the embryological ages during normal development. Detailed descriptions of embryological development in the mouse may be found in other sources, including Rugh (1968). Day $6\frac{1}{2}$ represents a stage shortly after implantation at about the time the mesodermal germ layer is beginning to appear. By day $10\frac{1}{2}$ embryos are in a period of major organogenesis. Concerning the reproductive organs specificially, the primordial germ cells migrate from the yolk sac endoderm to the dorsal mesenteries and coelomic angles between days 8 and 12. The gonad primordia begin to form by day $10\frac{1}{2}$, but the gonads remain undifferentiated until day 14 at which time testes and ovaries can be distinguished. Spermatogenesis becomes evident by day 14 and by day $14\frac{1}{2}$ interstitial tissue and germinal epithelium are apparent. Development of the ovary is similar, and by day 14 oogonia in mitosis can be observed. Secondary sex characters begin to differentiate by day 17.

Several studies have been concerned with evaluating the response of the fetal reproductive system to irradiation. These studies have included both histological and functional aspects and have indicated that the reproductive system of the fetal female can tolerate more radiation than that of adult females. In contrast to the findings in adult animals, fetal males are more drastically affected by irradiation than fetal females. Rugh and Jackson (1958) observed the adult reproductive capacity of mice which had been exposed between days $15\frac{1}{2}$ and $18\frac{1}{2}$ of gestation to x-ray doses of from 50 r to 200 r. Peak sensitivity of the ovary occurred on day $16\frac{1}{2}$ after 200 r. After day $16\frac{1}{2}$ the fetal ovary appeared to be quite radioresistant. Russell, Badgett and Saylors (1960), irradiated mice on days $\frac{1}{2}$ to $13\frac{1}{2}$ and observed that irradiation on days $11\frac{1}{2}$ and $13\frac{1}{2}$ exerted the greatest effect on the reproductive capacity of the females.

In the present study, four different periods of pregnancy were sampled. The dose of 80 r is the highest dose that is represented at all stages. With this dose, day $10\frac{1}{2}$ appears to be more radiosensitive than the other three ages. The mice that survived 160 r at $6\frac{1}{2}$ days appeared fully fertile. Normality of surviving embryos from irradiation at early embryological stages has been noted for other characteristics as well. A dose of 160 r appears to have a slightly greater effect at $14\frac{1}{2}$ days than at $17\frac{1}{2}$ days, and the same relationship appears to hold true following a dose of 320 r.

Among fetal males, the study of Rugh and Jackson (1958) indicated that the peak of radiosensitivity was at $15\frac{1}{2}$ days when an exposure of 200 r caused 45% of the males to be sterile, and fertile ones produced only 16% as many progeny as controls. With increasing embryological age fertility of males irradiated with 200 r improved towards control levels.

In the present study, at the lower doses, the response of the fetal testis appeared

to be similar to that of the fetal ovary. No effect on the adult male reproductive system was noted among the survivors until a dose of 80 r at day 10½, which reduced fertility to 81% of that of control males, again indicating that the 10½ day stage is the most radiosensitive. There was a small but insignificant decrease in reproductive performance of males that had received 160 r at day 6½ but in other characteristics such as postnatal survival, growth, and lifespan, these animals were comparable to controls. The 14½-day stage appeared to be more sensitive than the 17½-day stage as evidenced by reproductive performance following a dose of 160 r. At the former stage this dose reduced fecundity to 85% of that of controls, whereas at the latter stage, this dose did not affect fecundity, but did appear to affect fertility. Compared to the fetal ovary the fetal testis at 14½ days was less sensitive. Following irradiation at day 17½ the females appeared relatively more affected than the males at lower doses, but following the highest dose used, were less affected.

The present studies confirm the well-established fact that genetic differences influence reproductive capacity. Although significant genotypic differences were found for both number of litters and number of progeny, the interaction involving genotypes and levels of irradiation were not significant in all cases indicating that the different genotypes were responding in a similar way to the effects of embryonic irradiation upon postnatal reproductive performance. Differential genetic responses to irradiation have been noted for these mice for growth (Nash and Gowen, 1962) and lifespan (Nash and Gowen, 1965). A possible difference in response between inbred and hybrid mice was noted following a dose of 320 r on day 17½. Although all of the inbred progeny were sterile, 50% of the hybrid progeny were fertile. In Figure 3 the response of inbred and hybrid mice following irradiation at day 17½ is given. Results indicate that hybrids are less affected by doses of 160 r and 320 r, fecundity among hybrids remaining relatively normal until a dose of 320 r is reached. Heterosis in radiation response of the adult reproductive system has been noted previously by Haverland and Gowen (1960) and Ehling (1964). Differences in response of inbred and hybrid embryos may be a reflection of slight differences in embryological stage of development at the time of irradiation as well as a reflection of inherent differences in susceptibilities to radiation-induced effects on the reproductive system.

Summary

1. Lifetime reproductive performance was observed in mice that had been irradiated at different stages of embryological development. Three genetically differentiated inbred strains of mice, BALB, K, and S, and all their possible hybrids, including reciprocals, were used. Pregnant females were exposed to single whole-body 250 pkv x-ray dosages from 20 r to 320 r on 6½, 10½, 14½ and 17½ days gestation, as timed from the appearance of a vaginal plug. Efforts were made to obtain two mice of each sex and inheritance type for each of the irradiation dose-embryological age treatment combinations. No progeny or not sufficient progeny were obtained following irradiation with 160 r or 320 r at embryological ages of 6½, 10½, and 14½ days of age. At 75 days of age treated mice were outcrossed to mice of the Z strain, and matings were maintained for the lifetime of the treated mice.

2. Adult reproductive performance was found to be related both to the age of the embryo at the time of irradiation and the level of irradiation. The embryological ages in order of increasing sensitivity were $6\frac{1}{2}$, $17\frac{1}{2}$, $14\frac{1}{2}$, and $10\frac{1}{2}$ days. The incidence of sterile matings was pronounced only following 160 r or 320 r at $17\frac{1}{2}$ days. Significant effects on reproduction due to genotype and sex were observed at all ages. Survivors of irradiation at $6\frac{1}{2}$ days had normal fertility and fecundity. A dose of 80 r at $10\frac{1}{2}$ days reduced reproductivity to 76% of control values. Significant effects due to levels of irradiation were observed at both $14\frac{1}{2}$ and $17\frac{1}{2}$ days. Mice irradiated with 160 r at $14\frac{1}{2}$ days produced only 72% as many progeny as controls. Following irradiation with 160 r at $17\frac{1}{2}$ days, irradiated mice produced 83% as many progeny as controls. With a dose of 320 r at $17\frac{1}{2}$ days the incidence of sterility was 67% and progeny production fell to 21%

3. Genetic differences in the response of the fetal reproductive system were most evident following irradiation on day $17\frac{1}{2}$. Inbred progeny appeared to be more radiosensitive than hybrid progeny. The incidence of sterility after 320 r was 100% in inbreds compared to 50% for hybrids. Fecundity following 160 r was reduced in inbreds, but remained near normal in hybrids.

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